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Hyperproliferative Response of a Monoclonal Memory CD8 T Cell Population Is Characterized by an Increased Frequency of Clonogenic Precursors[1]

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Strong memory T cell responses result partly from the selection of Ag-specific clones during immunization. In this study, we show that a monoclonal CD8 T cell population expressing a unique TCR is heterogeneous in terms of clonogenic potential following activation under optimal conditions. More importantly, the frequency of clonogenic cells is strongly increased among Ag-experienced cells, indicating that these cells were either generated or selected during the in vivo primary response. Moreover, strong proliferative responses of primed cells result from this enhanced frequency, as proliferating naive and primed cells display the same cycling parameters, i.e., lag time and intermitotic interval. Hence, these results suggest that the clonogenic potential of individual cells is imprinted before Ag encounter and that clonogenic precursors are selected or generated following in vivo activation. ***The Journal of Immunology*****, 2002, 168: 2147–2153.**

Immunological memory is characterized by faster and more efficient secondary responses. This phenomenon has been attributed to modifications in the repertoire of primed cell populations. Indeed, immunization selects high-affinity clones (1–3) and leads to the accumulation of Ag-specific T cells (4–8). However, acquisition of new functional properties by individual memory T cells also contributes to the efficiency of secondary immune responses (5, 9).

The difficulty to identify the very rare naive Ag-specific T cells by the peptide/MHC-tetramer technology (7, 8) and the modifications in the primed T cell repertoire (1–3, 7, 8) concur to hamper functional analyses of memory T cell clones in normal mice. TCR-transgenic mice, with monoclonal T cell populations, have been used to compare naive and memory T cell responses to Ag. However, studies using TCR-transgenic models revealed heterogeneity in the functional capacities of individual cells within monoclonal populations. Indeed, even under conditions in which all TCR-transgenic T cells are activated by Ag, only a fraction is able to enter cell division (10, 11). It has also been observed that only a fraction of naive Ag-activated TCR-transgenic T cells is able to differentiate into cytokine producers (11–17). However, the frequency of cytokine-producing cells is enhanced during secondary antigenic challenge (14, 16–18). Importantly, previous cytokine gene expression can be genetically imprinted (13) and favors future capacity of reexpression by the cells (18).

We have used TCR-transgenic mice derived from the cytotoxic clone F5, recognizing the NP68 peptide (residues 366–374) from influenza nucleoprotein (19). i.p. immunization of F5 TCR-transgenic mice with NP68 peptide leads to the generation of long-lived primed $CD8^+CD44^{int}$ T cells (20, 21). In F5 mice, all naive $CD8^+CD44^{low}$ and primed $CD8^+CD44^{int}$ T cells are resting in vivo, express the transgenic TCR, and respond to peptide stimulation (17, 21).[4] However, using a combination of limiting dilution assays (LDA),[5] CFSE analysis of cell division patterns, and mathematical calculation of in vitro proliferative parameters, we show that the hyperproliferative capacity of the pure primed $CD8^+CD44^{int}$ T cell population observed in bulk cultures only relies on the enhanced frequency of cells capable to proliferate and generate clonal progeny. These results unveil two important features of F5 TCR-transgenic CD8 T cells. First, even in a monoclonal homogeneous model, functional abilities of individual cells are heterogeneous and immunization leads to an increase in the frequency of clonable cells. Second, individual naive and memory T cells that are able to proliferate show the same pattern of cell division in terms of cell cycle numbers, lag phase, and intermitotic interval.

Materials and Methods

Mice and immunizations

C57BL/10, F5 TCR-transgenic mice, and $Rag1^{-/-} \times$ F5 ($Rag^{-/-}$F5) mice were a gift from D. Kioussis (National Institute for Medical Research, London, U.K.) (22). All mice were bred in the institute's animal facility. Thymectomies were performed on 5- to 7-wk-old mice, which were then allowed to recover for at least 4 wk before immunization. Mice were immunized i.p., once or twice at a 24-h interval, with 50 nmol A/NT/60/68 influenza virus nucleoprotein peptide NP68 (residues 366–374) (Synt:em,

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[5] Abbreviations used in this paper: LDA, limiting dilution assay; DC, dendritic cell.

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Nîmes, France) in saline. Mice were primed 5–12 wk before experiments. All primed and naive F5 or Rag$^{-/-}$F5 mice were thymectomized.

Cell staining and FACS analysis

For flow cytometry analyses, spleen cells were stained as described (17). The following Abs were used: FITC-conjugated H57-597 (anti-TCR$\alpha\beta$) from Immunotech (Marseille, France); PE-conjugated H1.2F3 (anti-CD69) and MP6-XT22 (anti-TNF-α) and CyChrome-conjugated 53-6.7 (anti-CD8α) from BD Biosciences (Le Pont de Claix, France); and FITC-conjugated IM7.8.1 (anti-CD44) produced in our laboratory. CFSE labeling has been previously described (23). Cells were analyzed using the FACScan (BD Biosciences, Mountain View, CA) and CellQuest software (BD Immunocytometry Systems, San Jose, CA).

For sorting, spleen cells were purified by centrifugation on Ficoll-Hypaque layer (Cedarlane Laboratories, Hornby, Ontario, Canada). CD8 T cells from F5 splenocytes were further purified by negative depletion of CD4$^+$, Gr-1$^+$, Mac-1$^+$, and MHC-class II$^+$ cells. F5 or Rag$^{-/-}$F5 CD8 T cells were then stained as for surface marker expression and sorted on a FACStar^{Plus} (BD Biosciences) using CellQuest software (BD Immunocytometry Systems). Purity of isolated populations was routinely >95%.

Cell culture

Cells were cultured in DMEM (Life Technologies, Cergy-Pontoise, France) supplemented with 6% FCS (Life Technologies), 50 μg/ml gentamicin (Life Technologies), 10 mM HEPES, pH 7 (Sigma, L'Isle d'Abeau Chesnes, France), 2 mM L-glutamine (Life Technologies), 50 μM 2-ME (Sigma), and 5% of a murine IL-2 cell culture supernatant containing IL-2 (50–100 U/ml final). Antigenic stimulation was performed either by various concentrations of NP68 peptide, in the presence of irradiated (3000 cGy) syngeneic C57BL/10 splenocytes, or by graded numbers of irradiated (3000 cGy) syngeneic C57BL/10 splenocytes or dendritic cells (DCs), previously pulsed for 2 h at 37°C with 1 μM NP68 peptide. DCs were purified from C57BL/10 splenocytes by centrifugation on a metrizamide layer (Aldrich Chemical, L'Isle d'Abeau Chesnes, France) (24). DC preparations were 75–95% pure, as determined by coexpression of high levels of CD11c and MHC class II, and purified DCs were mature, as evidenced by expression levels of CD80/CD86 costimulatory molecules (data not shown). For the proliferation assays, 0.5 μCi (2 Ci/mmol) of [^{3}H]thymidine (Amersham Pharmacia Biotech, Orsay, France) was added during the last 8 h of the cultures.

Calculations of the proliferative parameters of CD8 T cell populations

A total of 6×10^4 CD8 T cells was stimulated in 1 ml of medium, in the presence of IL-2, with 1.5×10^6 irradiated (3000 cGy) syngeneic C57BL/10 splenocytes and 1 nM NP68 peptide. They were harvested at various time points and stained with CyChrome-conjugated 53-6.7 (anti-CD8α) from BD Biosciences, and a maximal number (ranging from 12,000 to 140,000) of CD8$^+$CFSE$^+$ events was acquired. Cell numbers in each (0–255) fluorescence channel were imported into ProFit software (Kagi, Berkeley, CA) and analyzed as described (25). Briefly, the series of log-normal Gaussian curves that best fits the CFSE profiles was determined using Levenberg-Marquardt method. Each Gaussian curve was then integrated to calculate the number of cells in the corresponding CFSE peak. This allows avoidance of over- or underestimations given by percentages between marker bounds that are due to nonsymmetrical overlapping between CFSE peaks. As cell numbers double at each division cycle, the number of cells in division peak i was divided by 2^i to get the precursor cell number necessary to generate them. Peak values obtained were plotted against their corresponding division number. Not all cells respond to stimulation equally, but as probability to enter cell cycle follows a normal distribution, plotting the size of cell cohorts performing a defined number of cell divisions in the starting population against the number of divisions yields a Gaussian curve. For a given culture time, the Gaussian curve that best fits the experimental data was then used to determine the mean division number that the starting population is able to perform. Plotting mean division numbers against time generates a linear relationship (see Fig. 5*A*) for which the inverse of the slope estimates the average division time. The intercept of the line with division one yields the average time the population of CD8 T cells took to enter the first cycle, which corresponds to the lag time.

LDA analysis

A graded number (ranging from 0.3 to 1000) of CD8 T cells was seeded in 96-well plates in 200 μl of medium containing IL-2 and diverse antigenic stimuli (see tables). For each CD8 T cell concentration, 24 wells were seeded. After 10–14 days of culture, the wells containing a clone were scored as positive. The frequency of clonable cells was calculated as described (4).

Results

Hyperproliferation of CD8 T cells from primed Rag$^{-/-}$F5 transgenic mice relies mostly on a higher frequency of clonable cells

i.p. immunization of F5 TCR-transgenic mice with influenza virus nucleoprotein peptide NP68 leads to the generation of long-lived hyperreactive CD8$^+$CD44^{int} T cells (20, 21). Following antigenic stimulation in bulk cultures, these cells proliferate more and proliferation is detected at lower peptide concentrations than with naive CD8 T cells. However, although ~95% of the naive CD8$^+$CD44^{low} and primed CD8$^+$CD44^{int} T cells from F5 mice bind the same quantity of fluorescent NP68-loaded H2-D^b tetramers,[4] we cannot formally rule out that, on a Rag-positive background, the improved response of the primed population could result, in part, from the selection of CD8 lymphocytes expressing only the NP68-specific transgenic TCR during immunization. Hence, we immunized F5 $\times$ Rag1$^{-/-}$ with the NP68 peptide. Lymphocytes from naive and primed Rag$^{-/-}$F5 mice were then cultured for 4 days in the presence of IL-2 and graded concentrations of NP68 peptide. As assessed by [^{3}H]thymidine incorporation, primed CD8 T cells from Rag$^{-/-}$F5 mice proliferate more and at lower peptide concentrations than naive CD8 T cells in bulk cultures (Fig. 1*A*).

Differences in the proliferative responses observed between naive and primed cells may be explained by a lower activation threshold or a higher frequency of responding cells. Alternatively, each responding cell may be less prone to cell death or have acquired improved cycling capacities. To address this last point, CD8 T cells from naive and primed Rag$^{-/-}$F5 mice were stained with CFSE, a dye that covalently couples with cellular proteins and allows the follow-up of cell division over time by flow cytometry. As shown in Fig. 1*B*, naive and primed CD8 T cells cultured for 3 days in the presence of 1 nM NP68 peptide perform the same number of cell cycles. The main difference between the two populations lies within the percentage of nondividing cells, which is always higher for naive than for primed cells. These data indicate that individual primed cells show the same cycling pattern as naive cells, and favor the hypothesis of a higher frequency of dividing cells within the pool of primed CD8 T cells. LDA experiments were used to directly assess this and revealed a much higher frequency of clonable cells within the primed population cultured with 10 nM NP68 antigenic peptide (Table I). Similar results were also found in the F5 background (Table I) and at 1 and 100 nM peptide concentrations (data not shown). These LDA results rule out the possibility that the higher percentage of nondividing naive cells observed in the CFSE experiments is due to a better survival of these cells. Indeed, LDA experiments that assess both capacities to proliferate and survive after antigenic stimulation reveal more differences between the two populations than the CFSE experiments. This suggests that, following activation, primed CD8 T cells have better survival capacities than naive CD8 T cells. Besides, as CD8 T cells from Rag$^{-/-}$F5 mice only express the transgenic TCR, the observed increased frequency is not due to the counterselection, during immunization, of cells expressing two antigenic specificities. Finally, the differences between individual CD8 T cells expressing the same TCR do not reside in the ability to be stimulated by Ag. Indeed, all naive and primed Rag$^{-/-}$F5 CD8 T cells down-regulate their TCR, up-regulate CD69, and nearly all produce TNF-α upon antigenic stimulation at the doses used to assess proliferation (Fig. 2). Thus, as indicated by these

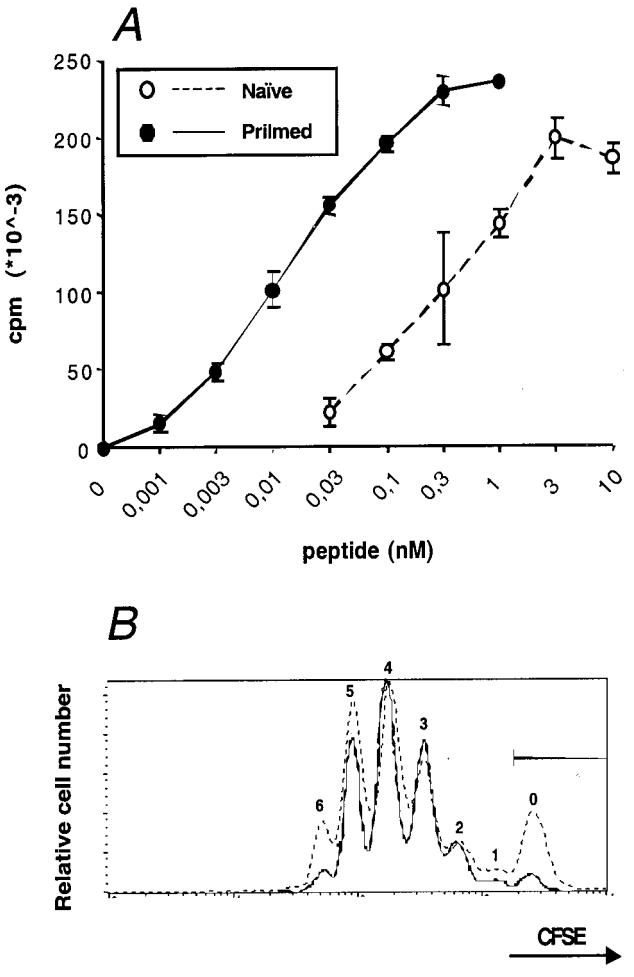

FIGURE 1. In vitro proliferation of CD8 T cells from naive and primed Rag$^{-/-}$F5 mice. *A*, [^{3}H]Thymidine incorporation by splenocytes from naive and primed Rag$^{-/-}$F5 mice cultured for 4 days with irradiated syngeneic feeder splenocytes, in the presence of IL-2, at the indicated NP68 peptide concentrations. One representative experiment of four is shown. *B*, Splenocytes from naive and primed Rag$^{-/-}$F5 mice were stained with CFSE and cultured with 1 nM NP68 peptide and irradiated syngeneic feeder splenocytes, in the presence of IL-2. The profiles of CFSE staining of CD8 T cells cultured for 3 days are shown. The percentage of nondividing cells was 12.5 and 3.4% for naive and primed cells, respectively. The number of cell cycles corresponding to each peak is indicated. One representative experiment of three is shown.

first activation steps, all naive and primed Rag F5 CD8 T lymphocytes are triggered by Ag, but only a fraction can generate a clonal progeny. This is not restricted to the F5 model, as a heterogeneity in the ability to proliferate after TCR stimulation has also been reported in the CD4$^+$ TCR-transgenic DO11.10 murine model (11).

Altogether, these results support the hypothesis along which the hyperproliferation of primed CD8 transgenic T cells observed in bulk cultures is mostly due to a higher frequency of cells entering division and generating clonal progeny, which we call clonogenic cells hereafter.

Sustained immunization leads to a further increase in the frequency of clonogenic CD8 T cells

To know whether a sustained immunization leads to a further increase in the frequency of clonogenic cells, we immunized F5 and Rag$^{-/-}$F5 mice twice at a 24-h interval with NP68 peptide (twice primed mice). We first looked at CD44 expression as the hyperproliferative response of primed CD8 T cells from F5 mice resides in the CD44^{int} compartment generated upon immunization (21). Fig. 3*A* shows that a second i.p. injection further increases the percentage of CD8 T cells expressing intermediate levels of CD44 6 wk after priming. Moreover, this immunization protocol leads to a further enhancement of the proliferative responses in bulk cultures of twice primed CD8 T cells (Fig. 3*B*). However, these cells did not perform additional cell cycles over a 3-day culture period, as compared with naive counterparts (Fig. 3*C*), but rather displayed a further increase in the frequency of clonable cells (Table I). In conclusion, sustained immunization leads to a further increase in the size of the CD8$^+$CD44^{int} hyperreactive primed population and to an increase in the frequency of clonogenic cells. Interestingly, this model may help in studying the immunization conditions necessary to generate a memory population in which all the cells have acquired clonogenic capacities.

Differences in the frequencies of clonogenic cells between naive and primed pure CD8 T cell populations are not reverted by optimal costimulation

It has been shown that memory T cells depend less on costimulation than naive T cells (26–28). Therefore, the differences in the frequencies of clonogenic cells observed between primed and naive CD8 T cells may merely reflect differences in the costimulation requirements of both populations. To address this question, we used DCs purified from syngeneic C57BL/10 mice and pulsed with NP68 peptide to assess the proliferation of naive and primed CD8 T cells. Naive (CD8$^+$CD44^{low}) and twice primed (CD8$^+$CD44^{int}) T cell populations from F5 or Rag$^{-/-}$F5 mice were FACS sorted and stimulated with graded numbers of NP68-pulsed splenocytes or DCs. As expected, purified DCs are very efficient in triggering CD8 T cell proliferation (Fig. 4, compare *A* and *B*). Indeed, as few as 500 DCs are able to elicit naive cell proliferation, while $>2 \times 10^4$ splenocytes are required to initiate a proliferative response. Interestingly, naive or primed pure populations from Rag$^{-/-}$F5 mice always show better proliferative capacities than the corresponding cells from the F5 mice. However, this cannot be explained by differences in the transgenic TCR expression, as the majority (≥95%) of CD8 T cells from F5 mice bind NP68-loaded H2-D^b tetramers with fluorescence levels equivalent to CD8 T

Table I. *Clonogenic capacities of TCR-transgenic CD8 T cell populations*[a]

Mice	Naive	Primed[b]	Twice Primed[c]
F5	379 ± 232 (n = 3)	14.8 ± 8.6 (n = 2)	9 ± 1.1 (n = 6)
Rag$^{-/-}$F5	61 ± 46 (n = 4)	9.9 ± 0.7 (n = 2)	4.2 ± 1.3 (n = 3)

[a] Serial dilutions of splenocytes from the indicated mice were cultured in the presence of IL-2, 10 nM NP68 peptide, and irradiated syngeneic splenocytes. The frequency of clonable cells was calculated as described in *Materials and Methods*. The results are expressed as the inverse of the frequency of clonable cells (mean ± SD of n independent experiments).
[b] Primed mice were immunized i.p. with 50 nmol NP68 peptide.
[c] Twice-primed mice were immunized i.p. twice at a 24-h interval with 50 nmol NP68 peptide.

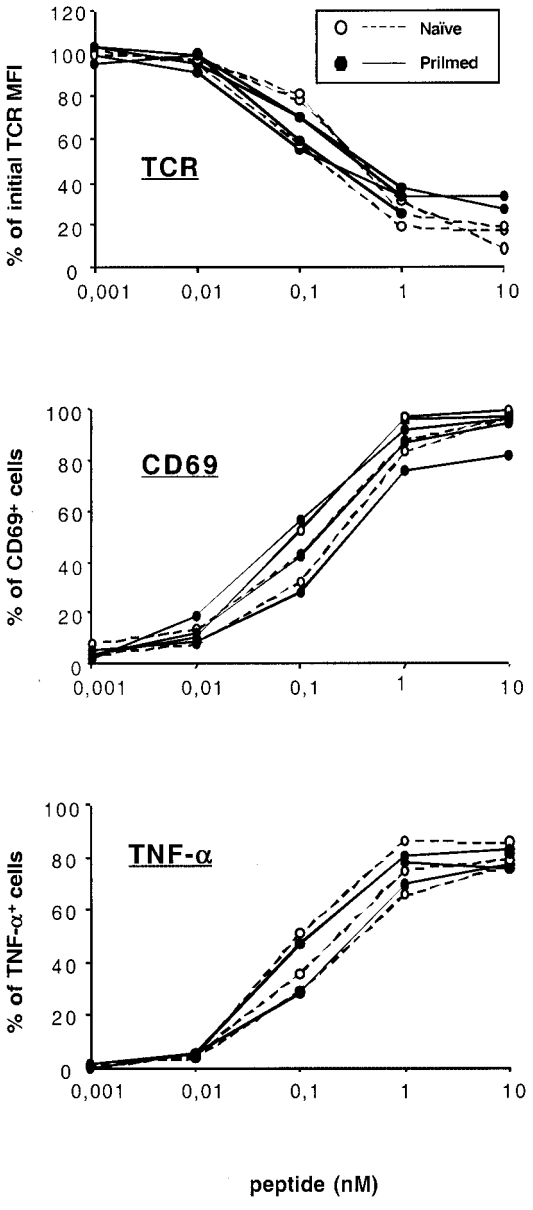

FIGURE 2. Short-term activation of CD8 T cells from Rag$^{-/-}$F5 mice. Splenocytes from naive or primed Rag$^{-/-}$F5 mice were stimulated for 5 h with the indicated peptide concentrations and subsequently stained for CD8, CD44, and the indicated molecules. The percentage of CD69^{+} and TNF-α-producing cells among naive CD8 and primed CD8 T cells is given. As all cells down-regulate TCR expression homogeneously, the results show the percentage of the mean fluorescence intensity of peptide-activated cells relative to cells cultured without peptide.

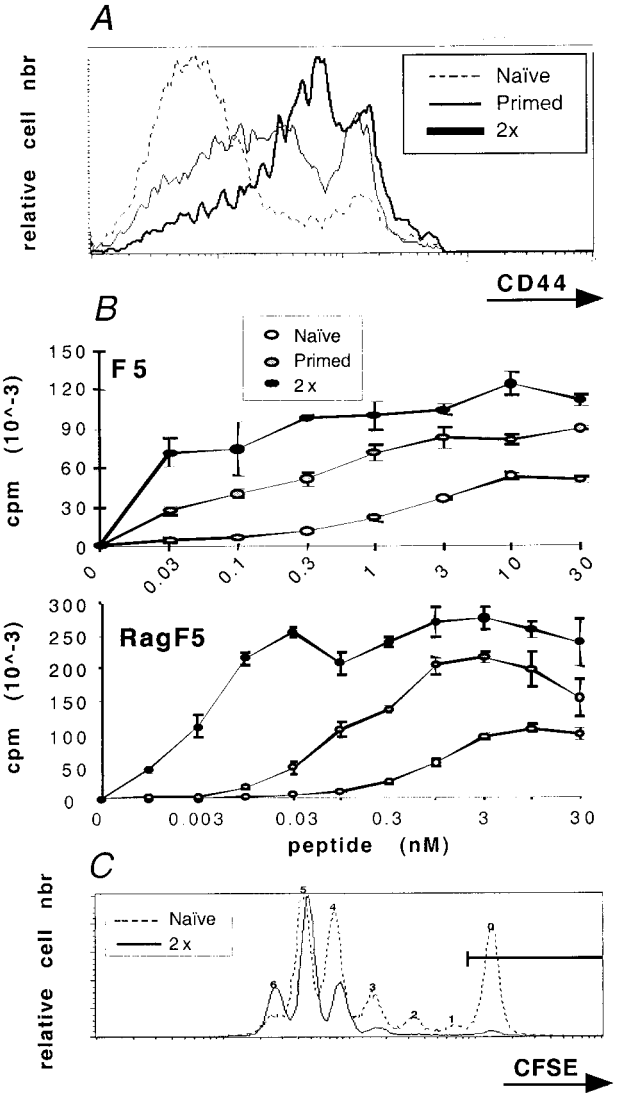

FIGURE 3. In vitro proliferation of CD8 T cells from mice immunized twice at a 24-h interval. *A*, CD44 expression pattern of CD8 splenocytes from naive F5 mice or F5 mice that have been immunized once (primed) or twice at a 24-h interval (2×). The respective percentages of CD44^{int} among CD8 T cells in these mice were 13.1, 33.1, and 53.4%. One representative experiment of four is shown. *B*, Splenocytes from F5 or Rag$^{-/-}$F5 mice, which have been once, twice, or not immunized, were cultured for 4 days in the presence of syngeneic irradiated feeder splenocytes and IL-2 at the indicated NP68 peptide concentrations. [^{3}H]Thymidine incorporation was assessed during the last 8 h of the culture. One representative experiment of three is shown. *C*, Splenocytes from naive and twice primed F5 mice were stained with CFSE and cultured with 1 nM NP68 peptide and irradiated syngeneic feeder splenocytes, in the presence of IL-2. The profiles of CFSE staining of CD8 T cells, cultured for 3 days, are shown. The percentages of nondividing cells were 23 and 2%, respectively, from naive and twice primed mice. The number of cell cycles corresponding to each peak is indicated. One representative experiment of two is shown.

cells from Rag$^{-/-}$F5 mice (data not shown). Still, as shown in Fig. 4, *A* and *B*, twice primed CD8^{+}CD44^{int} T cell populations always proliferate more and at lower antigenic dose than their CD8^{+}CD44^{low} naive counterparts, whether splenocytes or DCs are used as APCs. Finally, when using DCs as APCs, the hyperproliferative response of CD8^{+}CD44^{int} T cells was not due to a higher number of cell divisions, but rather to a higher percentage of dividing cells (Fig. 4*C*). LDA analysis performed on FACS-purified cell populations confirmed the presence of a higher frequency of clonable cells within the pure CD8^{+}CD44^{int} hyperreactive subset under optimal costimulation conditions (Table II).

Calculation of the proliferative parameters of naive and primed CD8 T cell subsets

Our CFSE experiments (Figs. 1*B*, 3*C*, and 4*C*) show that, within a given time interval, primed CD8 T cells do not seem able to perform more cell divisions than naive CD8 T cells. This indicates that, once they have entered cell cycle, naive and primed CD8 T cells proliferate with similar intermitotic intervals. However, it has

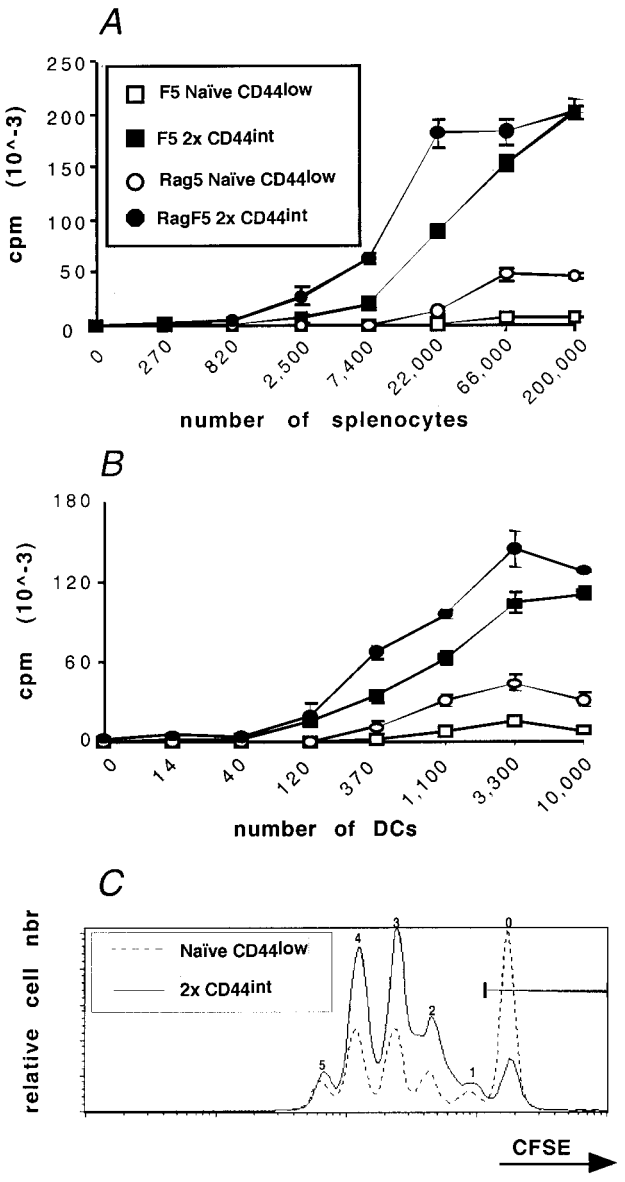

FIGURE 4. Hyperproliferation of $CD8^+CD44^{int}$-sorted T cells in the presence of optimal costimulation. *A* and *B*, $CD8^+CD44^{low}$ splenocytes from naive or $CD8^+CD44^{int}$ splenocytes from twice primed F5 or $Rag^{-/-}$F5 mice were FACS sorted and cultured for 4 days in the presence of IL-2 and graded numbers of irradiated syngeneic splenocytes (*A*) or DCs (*B*), pulsed with 1 μM NP68 peptide. [^{3}H]Thymidine incorporation during the following 14 h is shown. One representative experiment of two is shown. *C*, $CD8^+CD44^{low}$ splenocytes from naive or $CD8^+CD44^{int}$ splenocytes from twice primed $Rag^{-/-}$F5 mice were stained with CFSE and cultured for 62 h in the presence of IL-2 and irradiated syngeneic DCs pulsed with 1 μM NP68 peptide. The profiles of CFSE staining of CD8 T cells are shown. The percentages of nondividing cells were 33.9 and 8.5% for naive and twice primed cells, respectively. The number of cell cycles corresponding to each peak is indicated. One representative experiment of three is shown.

been observed in another transgenic model that the memory $CD4^+$ T cell population performs one more cell division than the naive $CD4^+$ T cell population, over a 3-day culture period (29). As the identification of the last division peak is difficult in some CFSE experiments, we chose to use the mathematical model published by others (25) to calculate the proliferation parameters (i.e., the cycling rate and the lag time of the response) of our naive and primed CD8 T cell populations (see *Materials and Methods*). Pure $CD8^+CD44^{low}$ and $CD8^+CD44^{int}$ T cell populations from naive and twice primed $Rag^{-/-}$F5 mice, respectively, were FACS sorted and labeled with CFSE. They were then cultured in the presence of IL-2, irradiated syngeneic splenocytes and 1 nM antigenic NP68 peptide. Results from two independent experiments are shown in Fig. 5 and show a good linear correlation between time and mean division numbers, indicating that, once they have entered the first

Table II. *Clonogenic capacities of pure CD8 T cell populations cultured in optimal costimulation conditions*[a]

Mice	Naive ($CD44^{low}$)[b]	Twice Primed ($CD44^{int}$)[c]
F5	249 ± 118	4.5 ± 2.7
$Rag^{-/-}$F5	75 ± 28	1.7 ± 0.4 (n = 2)

[a] Serial dilutions of sorted CD8 T cells from the indicated mice were cultured in the presence of IL-2 and irradiated syngeneic DCs pulsed with 1 μM NP68 peptide. The frequency of clonable cells was calculated as described in *Materials and Methods*. The results are expressed as the inverse of the frequency of clonable cells (mean ± SD of three independent experiments).

[b] $CD8^+CD44^{low}$ splenocytes from naive mice were sorted by FACS.

[c] Twice primed mice were immunized i.p. with 50 nmol NP68 twice at a 24-h interval at least 6 wk prior to FACS sorting of $CD8^+CD44^{int}$ splenocytes.

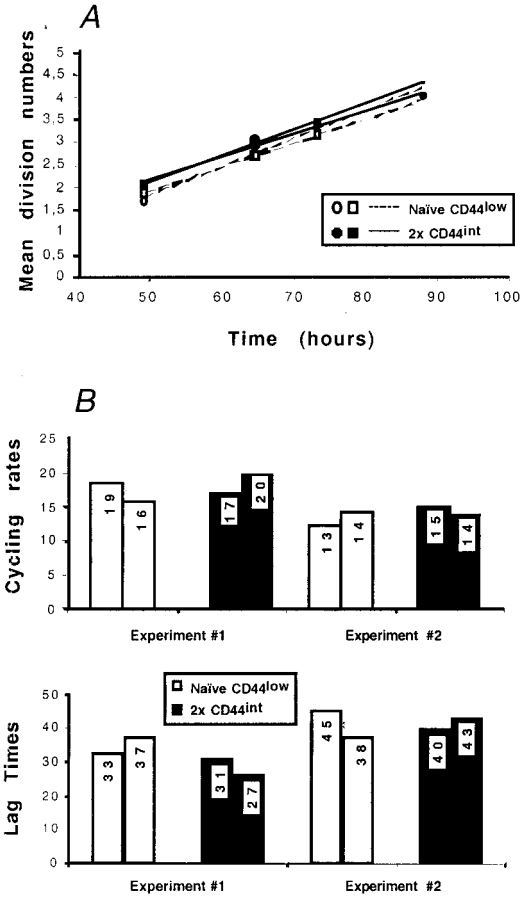

FIGURE 5. Proliferative parameters of naive and primed CD8 T cells. $CD8^+CD44^{low}$ splenocytes from naive or $CD8^+CD44^{int}$ splenocytes from twice primed $Rag^{-/-}$F5 mice were FACS sorted, stained with CFSE, and cultured for 4 days in the presence of IL-2, 1 nM NP68 peptide, and irradiated syngeneic splenocytes. At several time points, cells were harvested and CFSE profiles were analyzed. The proliferative parameters of the populations were calculated, as explained in *Materials and Methods*. *A*, Linear regression of two naive and twice primed populations from one representative experiment. *B*, Proliferative parameters of naive and twice primed populations from two independent experiments. The intermitotic intervals and lag time values are indicated.

division, both naive and primed CD8 T cells proliferate at a constant cycling rate during the assessed culture period (Fig. 5*A*). Results in Fig. 5 also reveal that naive and primed CD8 T cells from Rag$^{-/-}$F5 mice display similar lag times to enter the first division and intermitotic intervals. In conclusion, the frequency of dividing cells is the criterion distinguishing primed and naive populations. Indeed, although CD8 T cell populations from naive and primed Rag$^{-/-}$F5 mice both contain cells unable to proliferate upon antigenic activation, primed and naive individual CD8 T cells that enter division display the same proliferative capacities.

Discussion

This study shows that the hyperproliferation of primed CD8$^+$CD44^{int} F5 TCR-transgenic T cells is due to a higher frequency of cells that are capable of entering cell division upon antigenic challenge and to generate a clonal progeny. Results were obtained using purified populations of naive and primed CD8 T cells (Table II and Figs. 4 and 5) expressing a single antigenic specificity (i.e., from Rag$^{-/-}$F5 mice). Therefore, they reveal that a homogeneous clonal population of T lymphocytes in terms of *TCRA/TCRB* gene expression can display functional heterogeneity, and that immunization, even in a TCR-transgenic context, leads to the selection or generation of a subset of cells with clonogenic capacities.

Interestingly, not all primed CD44^{int} CD8 T cells are clonogenic precursors (Table II), although most of them have been Ag experienced. Indeed, >90% of these CD8$^+$CD44^{int} T cells proliferated, and all of them switched to an activated phenotype following priming (21).

A homogeneous population of lymphocytes with heterogeneous functional abilities raises the question of the nature of the differences between individual cells. It is possible that during positive selection of thymocytes, the recognition of different self peptides or the nature of the thymic APCs imprints the proliferative capacities within the offspring CD8 T cells and leads to a heterogeneous peripheral naive population of cells, with their DNA already prepared or not for replication (30, 31). In other words, the extent of thymic precursor proliferation or the frequency of peripheral reexposure to positively selecting ligands (32) may result in the progressive sensitization of the daughter cells to acquire proliferative abilities after subsequent foreign Ag encounter in the periphery. A similar phenomenon has been described for the cytokine production capacities of peripheral T cells (11, 13, 33). As positively selecting ligands drive the homeostatic proliferation undergone by naive T cells under lymphopenic conditions (32), it would be interesting to compare clonogenic capacities and the extent of homeostatic proliferation in several TCR-transgenic models. Alternatively, the capacity to enter cell division may be regulated by competition between individual CD8 T cells for extrinsic signals. Indeed, it has been shown that lymphocytes deprived of extrinsic signals, such as TCR triggering or cytokines, cease to utilize nutrients, leading to a cellular atrophy (34, 35) and delayed cell cycle entry upon retriggering (34–36). Furthermore, T lymphocytes compete in vivo for such extrinsic factors (34). Interestingly, we always found a higher frequency of clonable cells in naive populations from Rag$^{-/-}$F5 than from F5 mice. This is unlikely to be due to differences in the ability to bind Ag, as it was observed with pure F5 CD8$^+$CD44^{low} naive populations, >95% of which bind similar levels of NP68-loaded fluorescent H2-D^b tetramers (data not shown). Moreover, optimal costimulatory conditions did not reduce the differences observed (Fig. 4, *A* and *B*, and Table II). Thus, the differences in the frequencies of clonable cells between Rag$^{-/-}$F5 and F5 naive CD8 T cell populations might result from the level of in vivo exposure to extrinsic factors, for which competition should be less drastic in Rag$^{-/-}$F5 animals that do not have any B or CD4$^+$ T cells. The fact that ex vivo unstimulated naive CD8 T cells from Rag$^{-/-}$F5 mice are significantly larger than naive CD8 T cells from F5 mice favors this hypothesis (data not shown). Interestingly, increased clonogenicity of Rag$^{-/-}$F5 naive CD8 T cell populations may explain why in some TCR-transgenic models no differences were found in the proliferative capacities of naive and primed populations (37). Indeed, in that case, all cells in the naive population may already be imprinted with clonogenic capacities.

Our results show that individual naive cells that are dividing display identical proliferative capacities to the primed dividing cells but that primed populations contain a higher proportion of clonogenic cells. This is not due to differences in distribution in the different phases of the cell cycle of naive CD8$^+$CD44^{low} and primed CD8$^+$CD44^{int} F5 TCR-transgenic T cell populations that are identical, as >99% of cells in each subset are in G_0/G_1 (data not shown). Two models could explain these results. In a selective model, the naive cell population is heterogeneous. Following antigenic stimulation, the majority of cells acquire functional features such as TNF-α production, but only a fraction is predetermined to enter cell division and give a clonal progeny. These cells would expand while maintaining their clonogenic capacities, leading to a higher frequency of these cells in the resulting Ag-experienced population. This hypothesis is supported by the clonogenic difference observed between naive CD8 T cell populations from Rag$^{-/-}$F5 and F5 mice. It would also explain why there is no correlation between the size of a given clone in the naive and the primed repertoire (38). Alternatively, in an instructive model, all naive cells are able to proliferate and generate clonal progeny upon immunization. However, depending on the amount or duration of TCR triggering (39) or the context in which Ag is presented, some cells acquire immediate effector functions and die, while others proliferate and survive. Epigenetic imprinting of previous Ag-induced proliferation would then result in a higher frequency of clonogenic cells in this population. Both models would agree with the observation that a sustained immunization on 2 consecutive days further increases the frequency of cells capable of proliferating (Fig. 3 and Table I). Indeed, a second immunization further promotes the proliferation and expansion of adoptively transferred naive F5 CD8 T cells, already stimulated by peptide injection 24 h earlier (data not shown). These stochastic and instructive models are not exclusive, and both lead to the generation of a higher frequency of clonogenic cells following immunization. This phenomenon may have important consequences on the homeostasis of memory CD8 T cell clones. Indeed, while stimulating the same number of Ag-specific precursors, one would achieve different clonal burst with naive or memory cells following rechallenge. This phenomenon might be involved in the preferential restimulation of cross-reactive cells when they are Ag experienced (40, 41). It would also allow potent secondary responses from a smaller pool of Ag-specific memory clones.

It becomes clear from the present study and others (16, 17, 42) that the properties of memory populations rest on the frequencies of responding cells. Previous studies using either LDA or MHC-tetramer technology showed that immunization with pathogens leads to an increased frequency of Ag-specific cells (4–8). It was therefore concluded that the larger secondary responses were at least partly due to the higher frequency of Ag-specific cells within the total repertoire. We show in this study that besides this well-described phenomenon, immunization can also generate or select cells with clonogenic capacities within a given monoclonal Ag-specific population. The new heterogeneity at the clonal level that we demonstrate in this study in vitro cannot be measured in vivo,

as in these conditions nondividing activated cells are eliminated more efficiently and will not be detected. In conclusion, large secondary responses could be tailored by the combination of the increased frequencies of both Ag-specific clones and clonogenic cells among these clones.

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